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(54) Title: COMPOSITIONS FOR OSTEOGENESIS AND CHONDROGENESIS

(57) Abstract

A drug composition, characterized by containing BMP and hedgehog protein; and specifically, a drug composition for osteogenesis and chondrogenesis, that has dramatically superior osteogenesis and chondrogenesis performance relative to conventional drugs for bone and cartilage formation that contain BMP alone.

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DESCRIPTION
Compositions for Osteogenesis and Chondrogenesis

Field of the Invention

The present invention concerns a drug composition characterized by containing a bone morphogenesis factor; and specifically, concerns a composition for osteogenesis and chondrogenesis.

Background of the Invention

Bone morphogenetic proteins (BMP) are members of the TGF-β superfamily of proteins which are known to have activity in the growth and differentiation of cells and tissue. Many BMPs are active protein that cause undifferentiated mesenchymal cells of subcutaneous tissue or muscular tissue to differentiate into chondroblasts or osteoblasts, thereby stimulating osteogenesis or chondrogenesis. The genes that code for various BMPs have been isolated, including the genes encoding BMPs 1 through 18, GDFs 1-14, MP52, BIP, and HP269. See also: Science **242**, 1528-1534 (1988); Proc. Natl. Acad. Sci. USA **87**, 2220-2224 (1990); Progress in Growth Factor Research **1**, 267-280 (1989); National Publication [*Tokuhyo*] No. Hei 2[1990]-500241, National Publication [*Tokuhyo*] No. 3[1991]-503649, National Publication [*Tokuhyo*] No. Hei 3[1991]-505098, WO 91/18098, WO 92/05199 and WO 93/09229; Biochemical and Biophysical Research Communications **204**(2), 646-652 (1994); and The Bone **9**(3), 51-63 (1995)). The above disclosures of all of the above applications are hereby incorporated by reference..

Investigations have been carried out concerning methods for using the aforementioned BMPs in the treatment of loss or insufficient formation of bone and cartilage, but a rigorous method for clinical application has not yet been established.

The hedgehog ("HH") family is a group of proteins that has received attention as critical factors in morphogenesis. At present, there at least four types of hedgehog that are present among vertebrates, the sonic hedgehog (Shh), the desert hedgehog (Dhh), the Indian hedgehog (Ihh) and the tiggy-winkle hedgehog (twhh). Shh protein is of particular interest among vertebrates in terms of its function as a neural tube and somite ventralizing factor, and as a factor in determining the antero-posterior axis of limb buds. A functional analysis of this substance has been carried out (Yodosha, Experimental Medicine Supplement BioScience Terms Library, Development - Nerves, edited by Toshi-ichi Nakamura, p.38-39).

The participation of Shh and Ihh proteins in the regulation of skeletogenesis has also been documented (Dev. Biol. 172, 126-138 (1995), Nature 383, 407-413 (1996)). Disclosures of all of the above are hereby incorporated by reference. However, there is no knowledge concerning the osteogenesis or chondrogenesis action of hedgehog proteins.

Objects of the Invention

Bone and cartilage tissues are formed via a continuous process wherein bone and cartilage precursor cells or bone and cartilage invades into the region in which formation is to occur, followed by the growth and differentiation of the bone or cartilage precursor cells or bone and cartilage cells, along with the synthesis of bone and cartilage matrix. This process depends on the size of the region to be generated, but in general, a lengthy period of a few weeks to a few months is required. Consequently, the discovery of a drug that facilitates osteogenesis and chondrogenesis and can rapidly induce the formation of bone and cartilage tissue is strongly desired in order to aid in the recovery of persons needing repair or induction of bone and cartilage tissue.

Summary of the Invention

As a result of research concerning the effect of hedgehog protein, the inventors of the present invention et al. discovered that abnormal ossification was induced in nude mice when the mice were implanted with fibroblast cells into which the N-terminus domain (Shh-N) containing the active site of Shh had been introduced. This result showed that it was possible to use hedgehog protein in clinical applications as a novel factor for inducing or repairing hard tissues.

In addition, upon carrying out investigations into the action of hedgehog protein with respect to osteogenesis and chondrogenesis, the surprising discovery was made that osteogenesis and chondrogenesis performance is greatly improved when hedgehog protein is used in conjunction with BMP that has a bone morphogenesis effect. The present invention was perfected on the basis of this discovery.

Specifically, the present invention comprises a drug composition, characterized by containing BMP and hedgehog protein; and specifically, comprises a drug composition used for osteogenesis and chondrogenesis. This composition exhibits much higher levels of

osteogenesis and chondrogenesis performance in comparison to conventional drugs for osteogenesis and chondrogenesis containing BMP alone.

Brief Description of the Figures

Figure 1 is a diagram showing the effect of the introduction of Shh-N and BMP-2/-4 genes into osteoblasts and the effect of the products of the introduced genes on osteoblast differentiation based on APase activity in Example 1.

Figure 2 is a diagram showing the effect of Shh-N-CM and BMP-2/-4 on osteoblast induction based on APase activity in Example 2.

Figure 3 is a diagram showing the effect of Shh-N-CM and rhBMP-2 on osteoblast differentiation based on APase activity in Example 3. Figure 3A represents the change over time, Figure 3B represents the Shh-N concentration dependence and Figure 3C represents the rhBMP-2 concentration dependence.

Figure 4 is a diagram showing the effect (concentration dependence) of recombinant murine ("rm") Shh-N and rhBMP-2 on osteoblast differentiation based on APase activity in Example 4.

Figure 5 is a diagram showing the effect of Shh-N-CM and BMP-2/-4-CM on the differentiation of RMD-1 cells into chondrocytes in Example 5 using the amount of ^{35}S-sulfuric acid incorporated into proteoglycan as an indicator.

Figure 6 is a diagram showing the influence of the Shh-N-CM treatment time on the differentiation of RMD-1 cells into chondrocytes in Example 6 using the amount of ^{35}S-sulfuric acid incorporated into proteoglycan as an indicator.

Figure 7 is a diagram showing the effect of rmShh-N and rhBMP-2 on the differentiation of RMD-1 cells into chondrocytes in Example 7 (concentration dependence of rmShh-N), using the amount of ^{35}S-sulfuric acid incorporated into proteoglycan as an indicator.

Detailed Description of the Invention

A. Bone Morphogenetic Proteins

With regard to the bone morphogenesis proteins (BMPs) that can be used in the present invention, any substance can be used without particular restrictions, provided that it is a protein that acts on undifferentiated mesenchymal cells, causing these cells to

differentiate into chondrocytes and osteoblasts. However, human BMP manufactured by recombinant DNA technologies is desirable from the standpoint of producing large quantities of good quality material that is clinically safe from an immunological standpoint, etc. Specifically, a transformant (cells or microorganism) containing recombinant DNA including the base sequence coding for the human bone morphogenesis protein is cultured, and the recombinant human bone morphogenesis protein produced by these transformants is isolated and purified to prepare the recombinant human bone morphogenesis protein (rhBMP). Examples of these human bone morphogenesis proteins (rhBMPs) that can be cited are rhBMP-2, rhBMP-3, rhBMP-4 (also referred to as rhBMP-2B), rhBMP-5, rhBMP-6, rhBMP-7 (rhOP-1), rhBMP-8, rhBMP-9, rhBMP-12, rhBMP-13, rhBMP-15, rhBMP-16, rhBMP-17, rhBMP-18, rhGDF-1, rhGDF-3, rhGDF-5, rhGDF-6, rhGDF-7, rhGDF-8, rhGDF-9, rhGDF-10, rhGDF-11, rhGDF-12, rhGDF-14. For example, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 and BMP-7, disclosed in United States Patents 5,108,922; 5,013,649; 5,116,738; 5,106,748; 5,187,076; and 5,141,905; BMP-8, disclosed in PCT publication WO91/18098; and BMP-9, disclosed in PCT publication WO93/00432, BMP-10, disclosed in United States Patent 5,637,480; BMP-11, disclosed in United States Patent 5,639,638, or BMP-12 or BMP-13, disclosed in United States Patent 5,658,882, BMP-15, disclosed United States Patent 5,635,372 and BMP-16, disclosed in co-pending patent application serial number 08/715,202. Other compositions which may also be useful include Vgr-2, and any of the growth and differentiation factors [GDFs], including those described in PCT applications WO94/15965; WO94/15949; WO95/01801; WO95/01802; WO94/21681; WO94/15966; WO95/10539; WO96/01845; WO96/02559 and others. Also useful in the present invention may be BIP, disclosed in WO94/01557; HP00269, disclosed in JP Publication number: 7-250688; and MP52, disclosed in PCT application WO93/16099. The disclosures of all of these applications are hereby incorporated herein by reference. Also useful in the present invention are heterodimers of the above and modified proteins or partial deletion products thereof. These proteins can be used individually or in mixtures of two or more, and rhBMP-2 is preferred.

The BMP proteins that are used are preferably expressed by mammalian cells (for example, CHO cells), bacteria (for example, E. coli), and yeast cells. Although rhBMP-2 is an example of an rhBMP protein for which large-scale production and purification methods have been established, the other rhBMP proteins can also be used after

manufacturing and purifying them in the same manner (Progress in Growth Factor Research **1**, 267-280 (1989)).

The known purified rhBMP-2 is a dimeric protein with a molecular weight of about 30,000. Each of the monomers has a high-mannose sugar chain at residue Asn^{56} (Abstract Sixth Interactive Symposium of the Protein Society, San Diego CA (1992)).

The amount of BMP in the bone and cartilage forming compositions of the present invention can be any concentration at which bone and cartilage induction is manifested, but preferably, when rhBMP-2 is used, the amount is preferably 0.1 μg/mL or more, with 1-1000 μg/mL being more preferred and 1-300 μg/mL being additionally desirable.

B. Hedgehog Proteins

It has been documented heretofore that Ihh and Shh have similar biological activities in terms of their capacity for double limb induction (Science **273**, 613-622 (1996)), and that the activity of Shh resides entirely in the N-terminus domain (Shh-N) ((Curr. Biol. **5**, 791-796 (1995), Cell **81**, 313-316 (1995)). Thus, Shh, Ihh, or the N-terminus domain Shh-N, which is the active region of Shh, can be used as the hedgehog protein in the present invention.

The hedgehog proteins that have been manufactured by recombinant DNA technologies are preferred from the standpoint of obtaining large quantities of good quality raw material. Specifically, a transformant containing recombinant DNA that includes the base sequence encoding the hedgehog protein is cultured, and the recombinant hedgehog protein produced by these transformants is isolated and purified to prepare a crude recombinant hedgehog protein. Methods whereby physiologically active recombinant hedgehog protein is produced in large quantities using E. coli have been documented by numerous researchers (Curr. Biol. **5**, 791-796 (1995), Nature **375**, 322-25 (1995)). The Shh and Ihh genes are highly conserved across all species ranging from chickens to humans (Cell **75**, 1401-1416 (1993), Science **273**, 613-622 (1996), Cell **75**, 1417-1430 (1993), Gene Bank Accession Numbers L38517 and L38518), and so the animal types are unlimited for hedgehog proteins. In addition, the molecular weight of the protein is small in comparison to Shh and Ihh, and thus the active N-terminus domain Shh-N of Shh, which is that has similar physiological activity, is preferred.

The amount of hedgehog protein contained in the compositions of the present invention can be any concentration at which bone and cartilage induction is manifested, but when Shh-N is used, the amount is preferably 2 ng/mL or greater, with 5 ng/mL-500 μg/mL being more preferred, and 10 ng/mL to 10 μg/mL being additionally desirable.

C. Administration

The bone and cartilage forming composition of the present invention can be administered systemically or locally as a solution that contains BMP and HH, or the proteins can be held on an appropriate support, and can be administered to the regions where osteogenesis or chondrogenesis is desired. The cartilage forming composition of the present invention can contain components other than those indicated above. Specific examples of these optional components include bio-absorbable supports, stabilizers, preservatives, solubilizers, pH adjusters and thickeners. In addition, supplements that are effective in osteogenesis and chondrogenesis can also be included, examples of which include fibronectin and osteonectin. These optional components can be added by appropriate methods at an appropriate stage during preparation of the composition of the present invention.

In order to repair various types of bone and cartilage loss, or in order to add bone or cartilage, the bone and cartilage forming composition of the present invention is administered by any method to the area at which osteogenesis or chondrogenesis is needed. When in the form of a liquid, the composition can be administered by injection, when in the form of a paste or clay, the composition can be administered by implanting, or when held on an appropriate support, the composition can be administered by hardening and molding the material into the desired shape, and then implanting the material as a solid.

The therapeutic method includes administering the composition topically, systemically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or other connective tissue or other tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the compositions which may also optionally be included in the composition as described above, may alternatively or additionally, be

administered simultaneously or sequentially with the composition in the methods of the invention. The compositions may be administered using a carrier or matrix which may provide slow release of BMP and/or HH protein, as well as proper presentation and appropriate environment for cellular infiltration. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of carrier or matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalcium phosphate, hydroxyapatite, polylactic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. For injectable formulations, the carrier is preferably a viscous gel, such as a collagen or polymer. Carriers and matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalcium phosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

The bone and cartilage forming composition of the present invention can be used in organisms following methods known in the relevant fields, and to this end, appropriate use can be made depending on the application, region to be treated, and condition of the patient.

The bone and cartilage forming compositions of the present invention do not have to be used individually, and these substances can also be used in combination with other known implants. For example, the composition can be used in combination with a collagen film, or a polylactic acid film or Goretex film employing the GTR method. These materials are biocompatible films used for localization and immobilization of the composition.

Alternatively, the proteins of the present invention may be delivered through the use of appropriate vectors in gene therapy applications. In such use, the vectors encoding both BMP and HH may be transfected into the cells of a patient *ex vivo*, and the cells may be reintroduced into a patient. Alternatively, the vectors may be introduced into a patient *in vivo* through targeted transfection.

The efficacy of the drug composition of the present invention with respect to osteogenesis and chondrogenesis can be confirmed in the manner described below.

D. Assays for Activity

The activity with respect to osteogenesis can be evaluated 1) by investigating the degree of osteoblast differentiation and propagation in C3H10T1/2 cells (Growth Factors 9, 57-71 (1993)), an undifferentiated mesenchymal cell line that has been reported to have a capacity for differentiation into osteoblasts, and 2) by investigating the differentiation and manifestation of functionality in primary cultured osteoblasts isolated from the skulls of chicken embryos. In both cases, variation in alkaline phosphatase activity (APase) of the cells is a marker for differentiation, and is used as an indicator.

Activity with respect to chondrogenesis can be evaluated by investigating chondrocyte differentiation in RMD cells, a undifferentiated rat mesenchymal cell line having a capacity for differentiation into chondrocytes (J. Bone Miner. Res. **11**, 544-553 (1996). The degree of differentiation into chondrocytes can be measured using the incorporation of ^{35}S-sulfuric acid into the extracellular matrix via the synthesis of cartilaginous macromolecular proteoglycan (GAG) (J. Bone Miner. Res. **11**, 544-553 (1996). In addition, accumulation of cartilaginous macromolecular proteoglycan can be easily determined by staining the cells with Alcian blue at pH 1.0.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the composition, e.g. amount of bone or other tissue weight desired to be formed, the site of bone or tissue damage, the condition of the damaged bone tissue, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of BMP proteins in the composition. Generally, systemic or injectable administration will be initiated at a dose which is minimally effective, and the dose will be increased over a preselected time course until a positive effect is observed. Subsequently, incremental increases in dosage will be made limiting such incremental increases to such levels that produce a corresponding increase in effect, while taking into account any adverse affects that may appear. The addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also effect the dosage.

Progress can be monitored by periodic assessment of bone or tissue growth and/or repair. The progress can be monitored, for example, x-rays, histomorphometric determinations and tetracycline labeling.

Examples

Practical examples that demonstrate the bone and cartilage forming effects of the drug composition of the present invention are shown below. The present invention is not in any way limited by the practical examples presented below.

The method for producing Hedgehog and BMP proteins used in the practical examples is described below.

Preparation of chicken Shh-N recombinant virus and chicken Shh-N proteins

Shh cDNA was recovered by the RT-PCR method from the total RNA of stage 25 chicken embryos. Next, the region coding for the 200 amino acids at the N-terminus was amplified by PCR while inserting a stop codon at the 200th residue. The resulting DNA fragment was subjected to terminal blunting, and after ligating a Cla I adaptor, the fragment was subcloned into the chicken retrovirus vector RCAS (A) (J. Virol. **61**, 3004-3012 (1987)).

This viral DNA was then transfected using calcium phosphate into chicken embryo fibroblast (CEF) cells that were prepared from 11-day virus-free eggs (M egg line, Nisseiken, Yamanashi) (Mol. Cell. Biol. 7, 2745-2752 (1987)). The CEF cells were cultured in medium 199 (GIBCO, Gaithersburg MD, USA) containing 10% fetal bovine calf serum (FBS; Cell Culture Laboratories OH, USA).

The culture supernatant from the CEF cells transfected with the viral DNA contained Shh-N recombinant virus and chicken Shh-N protein. The viral particles present in the culture supernatant that was collected were concentrated by ultracentrifugation according to the method of Cepko et al. (Mol. Cell. Biol. **13**, 2604-2613 (1993)), and were subjected to infectivity experiments. The culture supernatant containing the chicken N-Shh protein (Shh-N-CM) was then used in a virus-free mouse or rat cell culture system. Culture supernatant from CEF cells containing the viral vector alone (RCAS-CM) was used as a control.

Preparation of mouse recombinant N-Shh (rmShh-N)

Mouse Shh cDNA containing the entire length of the coding sequence was obtained by RT-PCR from the total RNA of 10-day mouse embryos. Next, the 73-651 base sequence region of the mouse Shh cDNA sequence based on the recording of MacMahon (Gene Bank Accession Number X76290, Cell 75, 1417-1430 (1993)) was amplified using a primer pair containing the Sac I site. At this time, a stop codon was inserted after the base 851 of the sequence. The resulting DNA fragment was then digested with Sac I, and was subcloned into the pQE30 plasmid using the QIA Express Kit (QIA Express Kit, Qiagen, GmbH, Germany). The plasmid was then transformed into E. coli by a routine method, and mouse recombinant N-Shh with a histidine tag attached to the N-terminus was synthesized. This protein was then purified using a nickel-chelate resin column (Proc. Natl. Acad. Sci 88, 8972-8976 (1991)). The experimental methods were all carried out according to the protocol included in the kit (The QIA Expressionist).

Preparation of human BMP-2 recombinant virus and human BMP-2 protein (hBMP-2)

Human BMP-2 cDNA containing the entire coding sequence was cloned from the total RNA of human osteosarcoma cells using the RT-PCR method. This DNA fragment was sub-cloned into sub-group A and B RCAS vectors (RCAS(A), RCAS(B)) (J. Virol. 61, 3004-3012, (1987)) using the same method that was used in constructing the chicken N-Shh recombinant virus as described above.

Next, culture supernatant from CEF cells that had been transfected with the viral DNA was concentrated, and was used in infectivity experiments. The culture supernatant containing the hBMP-2 protein (hBMP-2-CM) was then used in a virus-free mouse or rat cell culture system.

Preparation of mouse BMP-4 recombinant virus and mouse BMP-4 protein (mBMP-4)

Mouse BMP-4 cDNA containing the entire coding sequence was cloned out of the total RNA of 16-day mouse embryos using the RT-PCR method. This DNA fragment was used, and was subcloned into subgroup A and B RCAS vectors (RCAS(A), RCAS(B)) (J. Virol. 61, 3004-3012, (1987)) using the same method that was used in constructing the chicken N-Shh recombinant virus as described above. The culture supernatant from CEF

cells transfected with the viral DNA was then concentrated and was used in infectivity testing. Culture supernatant containing mBMP-4 protein (mBMP-4-CM) was then used in a virus-free mouse or rat cell culture system.

Cell Culturing

Chicken embryo undifferentiated fibroblast (CEF) cells were isolated from 11-day virus-free embryos, and were cultured in Medium 199 (GIBCO, Gaithersburg MD) containing 10% fetal bovine serum (FBS, Cell Culture Laboratories, OH USA). The mouse fibroblast cell strain C3H10T1/2 Clone 8 was received from the Riken Cell Bank, and was subcultured with Eagle's Minimal Essential Medium (MEM, Nissui, Tokyo) containing 10% FBS). The rat undifferentiated fibroblast strain RMD-1 was subcultured in Ham's F-12 medium containing 10% FBS. The chondrocyte differentiation tests for the RMD-1 cells were carried out by culturing in Ham F-12 containing 5% FBS.

Example 1

The Shh-N and BMP genes were introduced into osteoblast cells using the recombinant viruses, and the effects of the products of the introduced genes on osteoblast differentiation were investigated. Primary culture osteoblast cells were isolated from the skulls of 18-day virus-free chickens according to the method of Gerstenfeld et al. (Dev. Biol. **122**, 49-60 (1987)). Culturing was carried out with alphaMEM [sic] containing 10% FBS. The cells were introduced into culture plates, and after 2 h, chicken Shh-N recombinant virus (Shh-N-RCAS(A)), human BMP-2 recombinant virus (BMP-2-RCAS(B)) and mouse BMP-4 recombinant virus (BMP-4-RCAS(B) were added to the media. Cells were infected with individual viruses, or were double-infected with the different subcloned Shh virus and BMP virus. Controls were produced that consisted of a group that was left uninfected (none) and a groups that were infected with vector alone (RCAS(A), RCAS(B)). After 6 days, the cells were harvested using trypsin. Some of the cells were subjected to immunocolorimetry with AMV-3C2 anti-virus antibody (Developmental Studies Hybridoma Bank, Baltimore, MD) and viral infection was confirmed in 95% or more of the cells. The remainder of the cells were introduced into 48-well plates (Corning NY, USA) at 30,000 cells/well, and culturing was continued for 7 days. The culture medium (CM) was changed every 3 days.

After completion of culturing, the APase activity was measured according to the method of Pacifici et al. (Exp. Cell Res. **195**, 38-46 (1991)). Specifically, cells were lysed by sonication in 0.2% Triton X-100 and 0.9% NaCl, and colorimetric determination was carried out based on the paranitrophenol produced when the supernatant was added to a reaction liquid containing paranitrophenol-2-phosphoric acid as the substrate. The amount of DNA from the cells was measured by a fluorescent dye method (Anal. Biochem. **122**, 338-344, Exp. Cell Res. **195**, 38-46 (1991)).

The APase activity is expressed in the figures with 1 unit representing the activity that generates 1 nmole of paranitrophenol in 1 min from 1 μg DNA. The results are shown in Figure 1. Only 1 unit/μg DNA or less of APase activity was detected in the None, RCAS(A) and RCAS(B) groups. The cells that had been infected with Shh-N-RCAS(A), BMP-2-RCAS(B) or BMP-4-RCAS (B) showed an increase in APase activity of 2-3 times in comparison to the control groups. Moreover, APase activity that was 8-11x the activity of the control groups was detected in the cells that were double-infected with Shh-N-RCAS(A) and BMP-2-RCAS(B) or BMP-4-RCAS(B). These results suggest that Shh-N and BMP-2/-4 act synergistically to promote the differentiation of osteoblasts, and that these compounds have good osteogenesis effects.

Example 2

The effects of Shh-N-CM and BMP-2/-4-CM on the induction of osteoblasts (undifferentiated mesenchymal cells) were investigated. C3H10T1/2 cells were introduced into 48-well plates (Corning, NY USA) at 30,000 cells per well, and after 24 h, Shh-N-CM, hBMP-2-CM and mBMP-4-CM were added to a concentration of 25%. Culturing was carried out for 7 days. The culture liquid and CM were changed every 3 days, and after culturing, the APase activity was measured following the same method as in Example 1.

The results are shown in Figure 2. In spite of the addition of the culture supernatant from the CEF cells into which the control virus vector had been inserted (RCAS-CM), the C3H10T1/2 cells exhibited no APase activity, which was used as a marker of osteoblast cells. However, Shh-N-CM, hBMP-2-CM and m-BMP-4-CM each individually showed induction of APase activity in C3H10T1/2 cells, although the effect was weak. In addition when Shh-N-CM and hBMP-2-CM or mBMP-4-CM were added simultaneously high APase activity was synergistically induced in the C3H10T1/2 cells.

The results of this experiment suggest that Shh-N and BMP-2/-4 act synergistically to promote the differentiation of undifferentiated mesenchymal cells into osteoblasts, and that these compounds have good osteogenesis effects.

Example 3

The concentration dependence and temporal effects of Shh-N-CM and rhBMP-2 on the differentiation of osteoblasts was investigated in C3H10T1/2 cells. C3H10T1/2 cells were introduced into 48-well plates (Corning NY, USA) at 30,000 cells per well, and after 24 h, Shh-N-CM was added at a concentration of 25%, recombinant human BMP-2 (rhBMP-2, obtained from Yamanouchi Seiyaku) was added in the amount of 1 μg/mL, or both were added, and culturing was carried out for 7 days. The APase activity was measured by the same method as in Practical Example 1 on the 2nd, 4th and 7th days of culturing. The results are shown in Figure 3A. In addition, various concentrations of Shh-N-CM were added to the culture medium in the presence and absence of 1 μg/mL of rhBMP-2, and the APase activity was measured on the 7th day. The results are shown in Figure 3B. In addition, various concentrations of rhBMP-2 were added to the culture medium in the presence and absence of Shh-N-CM at a concentration of 25%, and the APase activity was measured on the 7th day. The results are shown in Figure 3C.

The effects of Shh-N-CM and rhBMP-2 on the stimulation of APase activity in C3H10T1/2 cells was apparent on the 2nd and 4th day of culturing respectively, and the effects increased over time. In addition, a synergistic increase in the manifestation of APase activity was seen when both substances were added at the same time (Figure 3A). The effects of Shh-N-CM and rhBMP-2, as well as the synergistic action of both substances, increased in accordance with concentration (Figures 3B and 3C). The optimal concentration for Shh-N-CM was 25% (Figure 3B). The optimal concentration for rhBMP-2 was 1 μg/mL (Figure 3C).

Example 4

The concentration dependence of the effects of rmShh-N and rhBMP-2 on osteoblast differentiation of C3H10T1/2 cells was investigated. C3H10T1/2 cells were introduced into a 48 well plate (Corning, NY USA) at 30,000 cells per well, and after 24 h, rmShh-N was added in concentrations ranging from 10 ng/mL to 10 μg/mL in the presence and absence

of 1 μg/mL of rhBMP-2, and culturing was carried out for 7 days. After completion of culturing, the APase activity was measured by the same method as in Example 1.

The effect of Shh-N on APase activity in 10T1/2 cells is shown in Figure 4. Shh-N stimulated APase activity in accordance with concentration in the same manner as Shh-N-CM, and clear synergistic effects were seen in the presence of BMP-2.

Example 5

The effect of Shh-N-CM and BMP-2/-4-CM on the differentiation of RMD-1 cells into cartilage cells was investigated. RMD-1 cells were introduced at 400,000 cells/well into 24-well plates (Iwaki Garasu, Chiba) coated with type I collagen, and HAM F-12 medium containing 5% FBS was added. After 24 h, Shh-N-CM, hBMP-2-CM and mBMP-4-CM were added individually or in conjunction to a concentration of 25%, and culturing was carried out. The medium was changed after 48 h, and culturing was then continued without adding CM. After 6 days, the amount of proteoglycan synthesis, which is an indication of chondrocyte differentiation, was measured by the incorporation of ^{35}S-sulfuric acid into the cetyl pyrimidine chloride [sic; probable error for "cetyl pyridinium chloride"] precipitate fraction (J. Bone Miner. Res. **11**, 544-553, (1996)). Specifically, 1.5 μCi of ^{35}S-sulfuric acid was added per well, and labeling was carried out by incubation for 6 h. The amount of ^{35}S-sulfuric acid incorporated into the proteoglycan of the cell layer and the amount contained in the culture supernatant were then measured.

The results are shown in Figure 5. RMD-1 cells exhibited almost no differentiation into chondrocytes, although culture supernatant was added from CEF cells into which the control vector had been introduced (RCAS-CM). However, Shh-N-CM, hBMP-2-CM and mBMP-4-CM all stimulated chondrocyte differentiation of RMD-1 cells when used individually. In addition, high synergistic levels of activity in terms of stimulating chondrocyte differentiation were seen when Shh-N-CM and hBMP-2-CM or mBMP-4-CM were added simultaneously. This experiment demonstrates that chondrocyte differentiation of RMD-1 cells is synergistically stimulated by Shh-N and BMP, and indicates that these substances have good chondrogenesis effects.

Example 6

The influence of Shh-N-CM treatment time on the differentiation of RMD-1-cells into chondrocytes was investigated. RMD-1 cells were introduced at 400,000 cells/well into 24-well plates (Iwaki Garasu, Chiba) coated with type I collagen, and HAM F-12 medium containing 5% FBS was added. After 24 h, culturing was carried out while treating with 25% Shh-N-CM for 2 days, 4 days or 6 days in the presence and absence of 200 ng/mL of rhBMP-2. The synthesis of proteoglycan, which is an indicator of chondrocyte differentiation, was measured by the incorporation of ^{35}S-sulfuric acid into the cetyl pyridinium chloride precipitate fraction.

This experiment demonstrates that the induction of differentiation of RMD-1 cells into chondrocytes is greatly accelerated when Shh-N is present for the initial 2 days, and that good synergistic effects can be obtained when this substance is used in conjunction with BMP-2.

Example 7

The effect of rmShh-N concentration on the differentiation of RMD-1 cells into chondrocytes was investigated. RMD-1 cells were introduced at 400,000 cells/well into 24-well plates (Iwaki Garasu, Chiba) coated with type I collagen, and HAM F-12 medium containing 5% FBS was added. After 24 h, concentrations of rmShh-N ranging from 10 ng/mL to 10 μg/mL were added in the presence or absence of 200 ng/mL of rhBMP-2, and culturing was carried out. After 48 h, the culture medium was changed, whereupon culturing was continued for an additional 7 days without adding recombinant protein. The synthesis of proteoglycan, which is an indication of chondrocyte differentiation, was measured based on the incorporation of ^{35}S-sulfuric acid into the cetyl pyridinium chloride precipitate fraction. rmShh-N increased proteoglycan synthesis in accordance with increasing concentration, and remarkably high levels of proteoglycan synthesis were seen in the presence of BMP-2. (Figure 5)

The results described above demonstrate that the drug composition of the present invention has good osteogenesis and chondrogenesis effects due to its capacity for dramatically stimulating the differentiation of bone and cartilage precursor cells into bone and cartilage cells, and for promoting the differentiation of osteoblast cells themselves.

Effect of the Invention

The implants of the present invention can rapidly induce osteogenesis and chondrogenesis *in vivo*, and can form (regenerate) bone and cartilage tissue. Consequently, the compositions can be used in patients in accordance with methods known in the relevant fields in order to repair various types of bone and cartilage loss resulting from injury, illness or congenital condition.

The transplant of the present invention can be used in a number of fields, for example, for repairing areas deficient in bone or cartilage resulting from accident, disease, congenital condition or various types of surgery, for accelerating the healing of various types of bone fractures, for forming bone around implants such as artificial teeth roots, for accelerating the fixation of artificial implants, for accelerating spine fusions and for regenerating or filling-in bone or cartilage in the field of orthopedic medicine, for example, in leg elongations. The invention can also be used in the field of plastic surgery in order to fill in bone or cartilage, or in the field of dentistry for repairing cavities, for regenerating alveolar bones, for increasing bone mass to repair cementum and for use with implants.

CLAIMS

1. A drug composition, characterized by containing hedgehog protein and bone morphogenesis factor.

2. The drug composition described in Claim 1, which is used for osteogenesis and chondrogenesis.

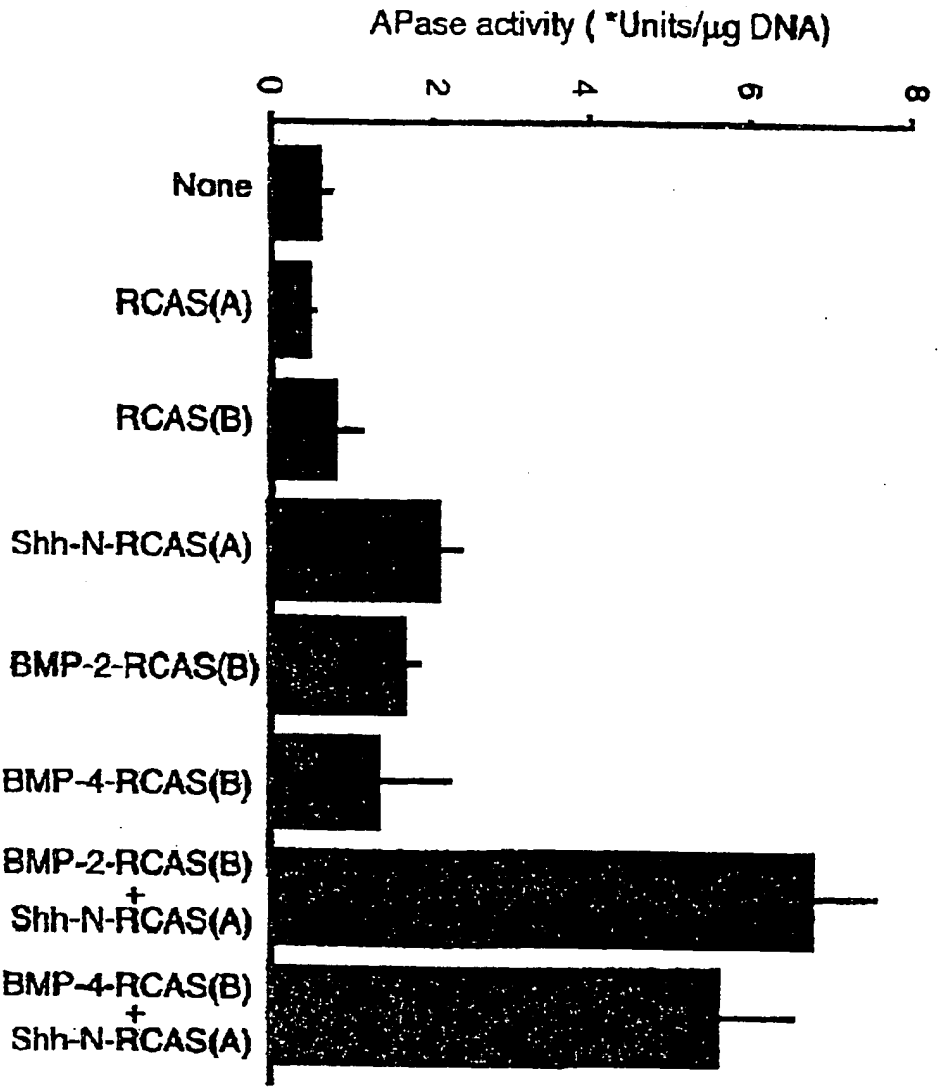
ALPase activity (*Units/μg DNA)
0
2
4
6
8
None
RCAS(A)
RCAS(B)
Shh-N-RCAS(A)
BMP-2-RCAS(B)
BMP-4-RCAS(B)
BMP-2-RCAS(B) + Shh-N-RCAS(A)
BMP-4-RCAS(B) + Shh-N-RCAS(A)

Fig.1

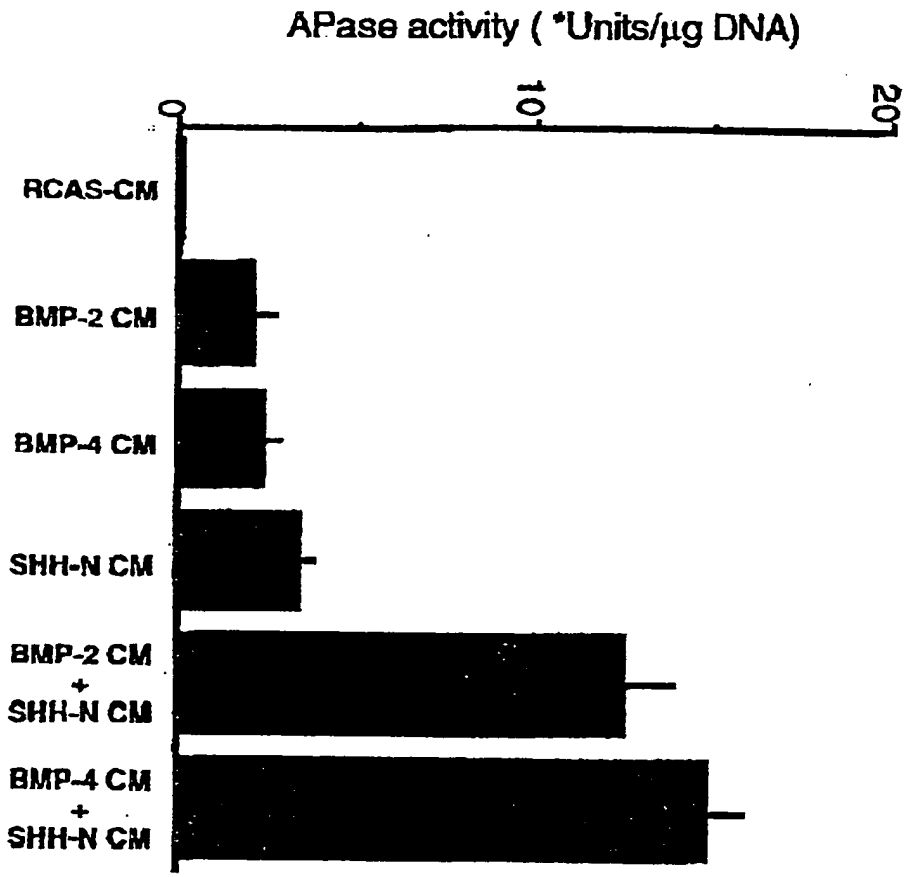
APase activity (*Units/μg DNA)
0
10
20
RCAS-CM
BMP-2 CM
BMP-4 CM
SHH-N CM
BMP-2 CM + SHH-N CM
BMP-4 CM + SHH-N CM

Fig. 2

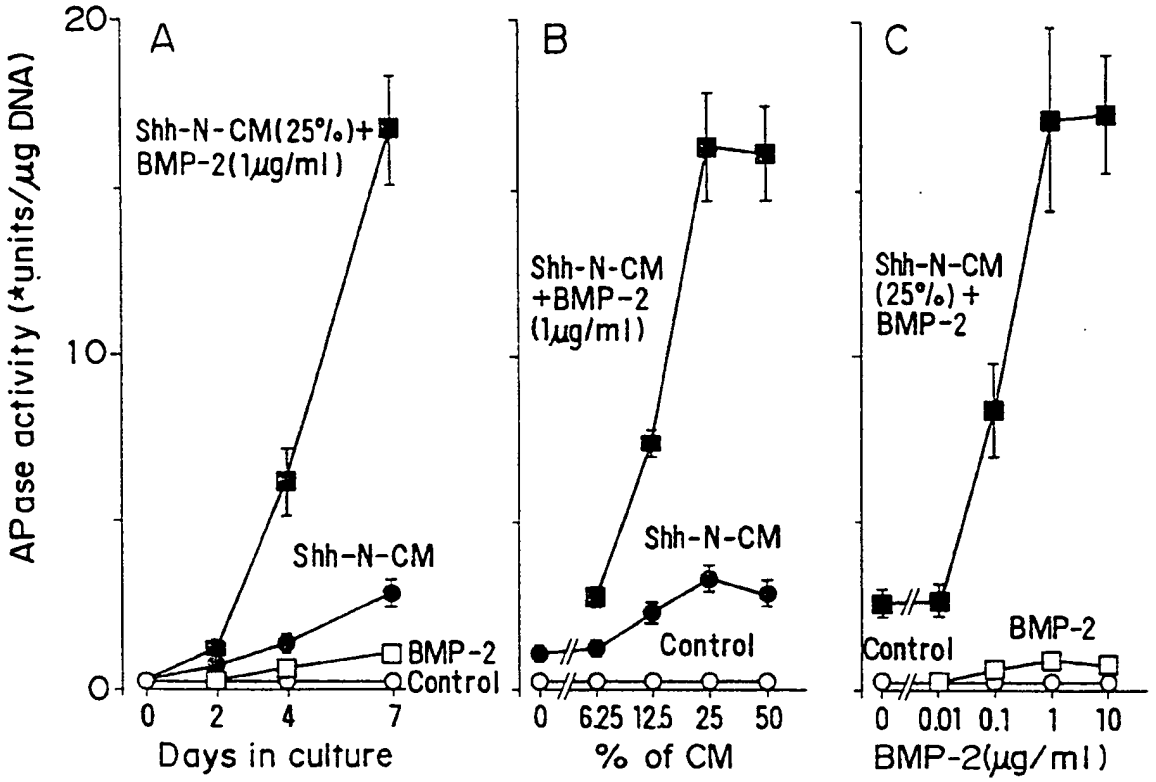
20
10
0
APase activity (*units/μg DNA)
A
Shh-N-CM(25%)+
BMP-2(1μg/ml)
Shh-N-CM
BMP-2
Control
0 2 4 7
Days in culture
B
Shh-N-CM
+BMP-2
(1μg/ml)
Shh-N-CM
Control
0 6.25 12.5 25 50
% of CM
C
Shh-N-CM
(25%)+
BMP-2
Control
BMP-2
0 0.01 0.1 1 10
BMP-2(μg/ml)

Fig.3

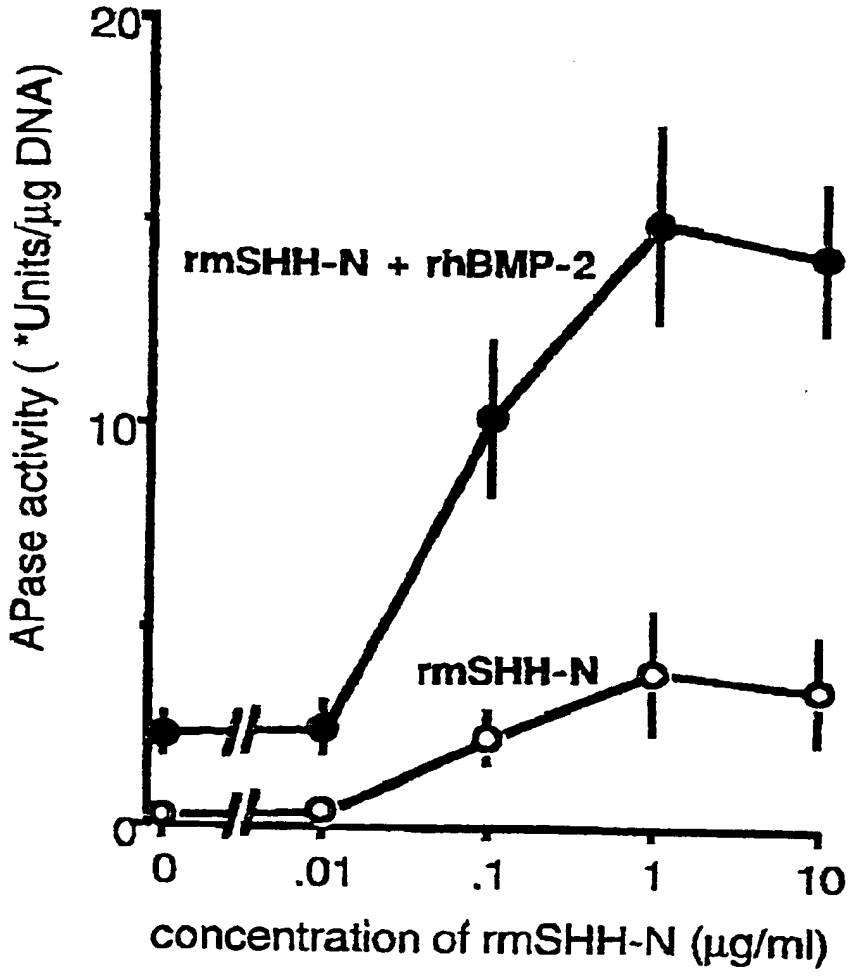
20
10
0
APase activity (*Units/µg DNA)
rmSHH-N + rhBMP-2
rmSHH-N
0
.01
.1
1
10
concentration of rmSHH-N (µg/ml)

Fig. 4

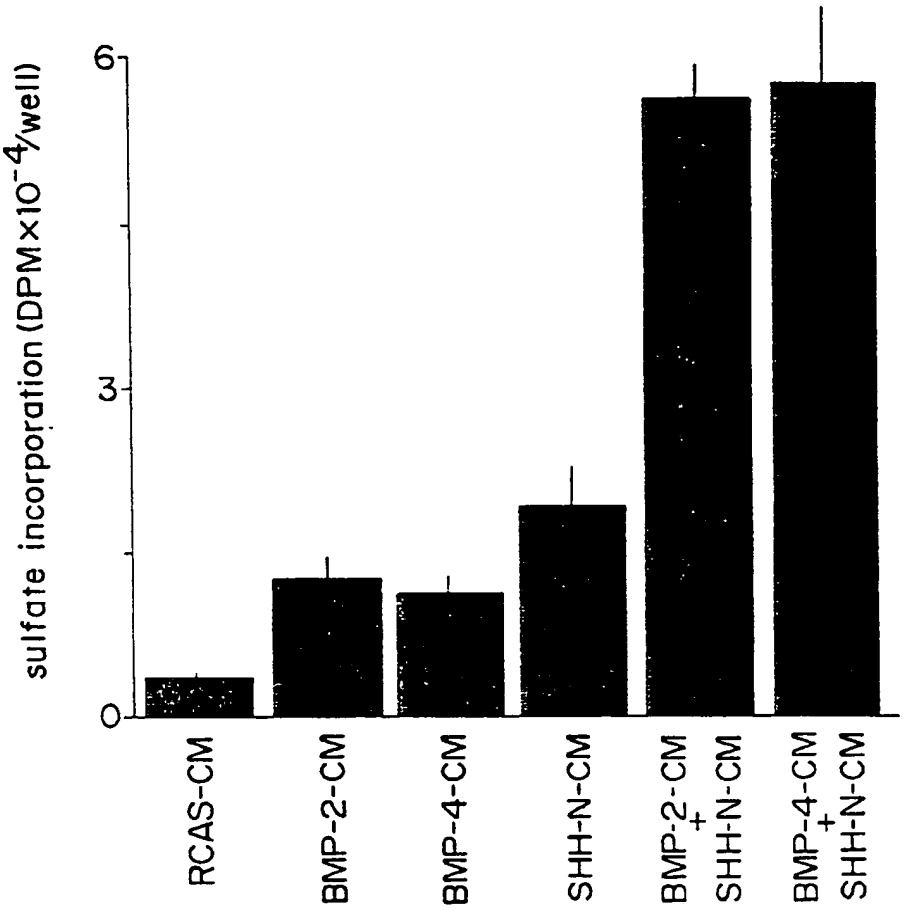
sulfate incorporation (DPM×10⁻⁴/well)
6
3
0
RCAS-CM
BMP-2-CM
BMP-4-CM
SHH-N-CM
BMP-2-CM + SHH-N-CM
BMP-4-CM + SHH-N-CM

Fig. 5

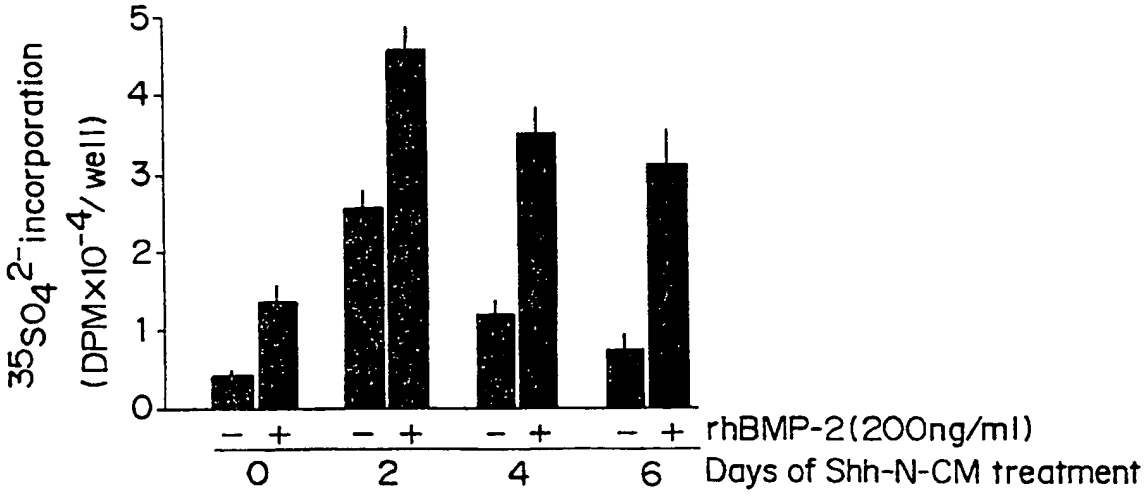
35SO4 2- incorporation (DPM×10⁻⁴/well)
5
4
3
2
1
0
− + − + − + − + rhBMP-2(200ng/ml)
0 2 4 6 Days of Shh-N-CM treatment

Fig. 6

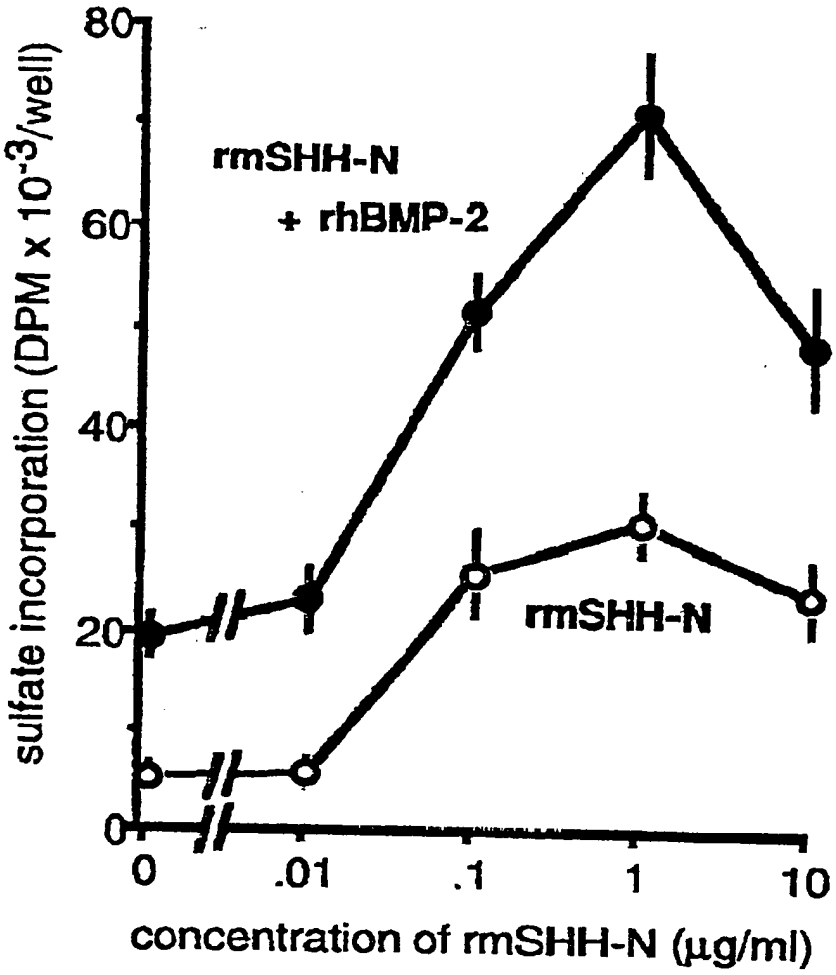
80
60
40
20
0
sulfate incorporation (DPM x 10^{-3}/well)
rmSHH-N
+ rhBMP-2
rmSHH-N
0
.01
.1
1
10
concentration of rmSHH-N (μg/ml)

Fig. 7

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/JP 98/00112

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 //(A61K38/18.38:18)

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimumcocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NAKAMURA ET AL.: "Induction of osteogenic differentiation by hedgehog proteins" BIOCHEM. BIOPHYS. RES. COMMUN., vol. 237, no. 2, 18 August 1997, pages 465-469, XP002064377 *see the whole document* ---	1,2
Y	BITGOOD ET AL.: "Hedgehog and and BMP genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo" DEVELOPMENTAL BIOLOGY, vol. 172, no. 1, 1995, pages 126-138, XP002064378 *see in particular p. 132, right column, 1st section, and p.136-137 section "hedgehogs and Bmps " * --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
8 May 1998	26/05/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/JP 98/00112

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KAWAKAMI ET AL.: "BMP signaling during bone pattern determination in the developing limb" DEVELOPMENT, vol. 122, no. 11, 1996, pages 3557-3566, XP002064379 * see the abstract and page 3564 *	1,2
Y	US 5 393 739 A (BENTZ HANNE ET AL) 28 February 1995 * claims and col. 10, lines 20- 53 *	1,2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]ional Application No
PCT/JP 98/03112

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5393739 A	28-02-1995	AU 651421 B	21-07-1994
		AU 9141991 A	25-06-1992
		CA 2071912 A	31-05-1992
		EP 0513334 A	19-11-1992
		WO 9209697 A	11-06-1992